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Implication of ZW10 in membrane trafficking between the endoplasmic reticulum and Golgi

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ZW10, a dynamitin-interacting protein associated with kinetochores, is known to participate directly in turning off of the spindle checkpoint. In the present study, we show that ZW10 is located in the endoplasmic reticulum as well as in the cytosol during interphase, and forms a subcomplex with RINT-1 (Rad50-interacting protein) and p31 in a large complex comprising syntaxin 18, an endoplasmic reticulum-localized t-SNARE implicated in membrane trafficking. Like conventional syntaxin-binding proteins, ZW10, RINT-1 and p31 dissociated from syntaxin 18 upon Mg^{2+}-ATP treatment in the presence of NSF and α-SNAP, whereas the subcomplex was not disassembled. Overexpression, microinjection and knockdown experiments revealed that ZW10 is involved in membrane trafficking between the endoplasmic reticulum and Golgi. The present results disclose an unexpected role for a spindle checkpoint protein, ZW10, during interphase.
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Introduction

The spindle checkpoint prevents sister-chromatid segregation until all kinetochores form stable bipolar microtubule attachments (for reviews see Millband *et al*, 2002; Cleveland *et al*, 2003). ZW10 was discovered as a protein that is required for faithful chromosome segregation in *Drosophila* (Williams *et al*, 1992, 1996; Williams and Goldberg, 1994). ZW10, which is conserved across large evolutionally distances (Starr *et al*, 1997), interacts with dynamitin, the p50 subunit of the dynactin complex (Echeverri *et al*, 1996), and facilitates the recruitment of the dynein–dynactin complex to the kinetochores (Starr *et al*, 1998). ZW10, together with ROD, is involved in the production and/or regulation of the force responsible for poleward chromosome motion (Savoian *et al*, 2000). Recent studies revealed that ZW10 and ROD are spindle checkpoint proteins (Chan *et al*, 2000), and that their transport away from kinetochores mediated by dynein leads to termination of the spindle checkpoint (Howell *et al*, 2001; Wojcik *et al*, 2001).

We have previously reported that syntaxin 18, a target membrane-associated SNAP receptor (t-SNARE), is localized in the endoplasmic reticulum (ER), and is involved in membrane trafficking between the ER and Golgi (Hatsuzawa *et al*, 2000). t-SNAREs form a tight complex with vesicle-associated SNAREs (v-SNAREs), which leads to membrane fusion (for reviews see Lin and Scheller, 2000; Wickner and Haas, 2000; Jahn *et al*, 2003). After membrane fusion, the *cis* (same membrane) SNARE complex is disassembled by ATPase NSF with the aid of α-SNAP in an Mg^{2+}-ATP-dependent manner. Several features of syntaxin 18 are similar to those of Ufe1p, a yeast ER SNARE that is implicated in retrograde transport from the Golgi to the ER (Lewis and Pelham, 1996) and ER membrane fusion (Patel *et al*, 1998).

In the present study, we show that syntaxin 18 forms a complex with ZW10, RINT-1 and p31 in addition to conventional syntaxin-binding proteins. The results of functional analyses suggest a novel role for ZW10, that is a role in membrane trafficking between the ER and Golgi.

Results

Identification of syntaxin 18-associated proteins

In an effort to elucidate the function of syntaxin 18, a t-SNARE located in the ER, we attempted to determine the composition of syntaxin 18 complexes. To this end, we raised monoclonal antibodies (mAbs) to syntaxin 18 and prepared a resin bearing one of the Abs named clone 2A6. Triton X-100 extracts of rat liver total membranes were mixed with the Ab-coupled resin, and the bound proteins were eluted, separated by SDS–polyacrylamide gel electrophoresis (PAGE), digested in gels and subjected to sequence analysis. The partial sequences of seven proteins in addition to syntaxin 18 were determined (Figure 1A). Four proteins (rSec22b, SNAP-23, α-SNAP and rSly1p) were conventional syntaxin-binding proteins, and the other three (p31, RINT-1 and ZW10) were new ones. p31 is encoded by an EST clone (AF220052), and its function was totally unknown. In the course of this study, however, three papers were published that describe the identification of yeast Use1p/Slt1p, an unconventional ER SNARE that interacts with Ufe1p (Belgareh-Touze *et al*, 2003; Burri *et al*, 2003; Dilcher *et al*, 2003). Sequence comparison

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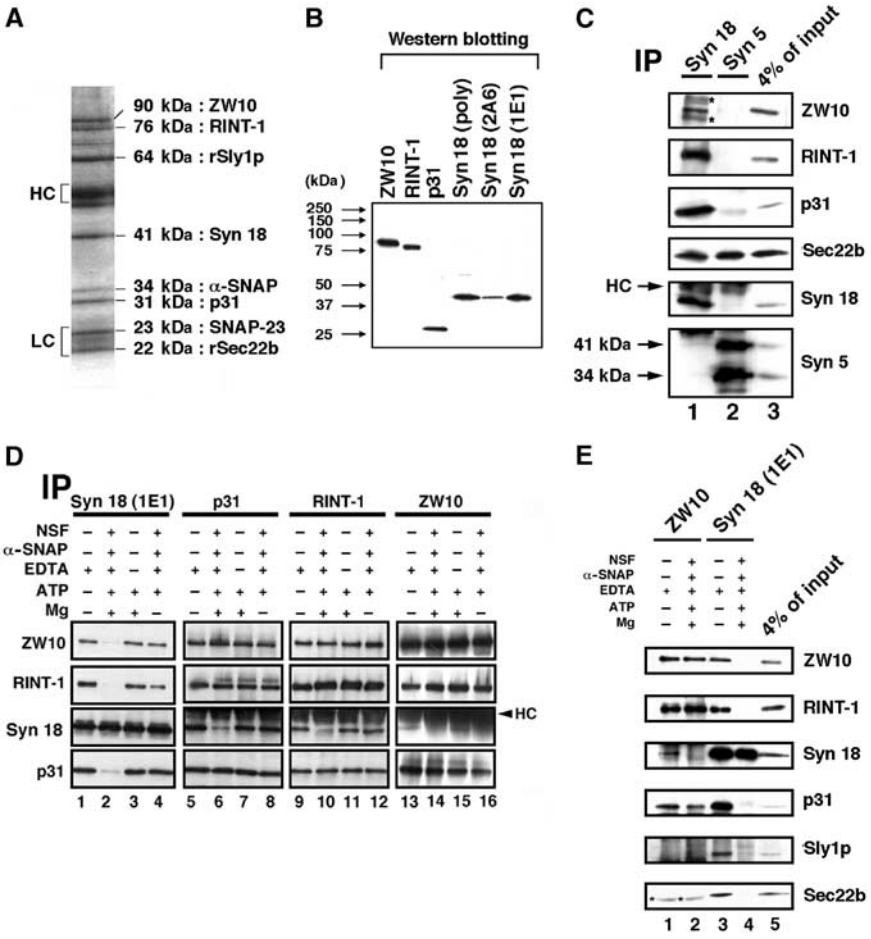

Figure 1 Identification of syntaxin 18-containing complexes. (**A**) Immunoisolated syntaxin 18 and its binding proteins were resolved by SDS–PAGE on a 7–15% gradient gel, and their partial sequences were determined. Labels to the right indicate their estimated molecular masses and identities. HC and LC denote immunoglobulin heavy chain and light chain, respectively. (**B**) 293T cell lysates (20 μg each) were subjected to SDS–PAGE on an 8% gel and immunoblotted with the indicated Abs. (**C**) Triton X-100 extracts of 293T cells (750 μl) were immunoprecipitated with a polyclonal Ab to syntaxin 18 (lane 1) or syntaxin 5 (lane 2). The precipitated proteins were separated by SDS–PAGE on a 10% gel and analyzed by immunoblotting with the indicated Abs. We also analyzed 4% of the lysate (lane 3). Asterisks indicate nonspecific bands. A faint band with mobility similar to that of p31 (lane 2) represents immunoglobulin light chain. (**D**) Triton X-100 extracts (750 μl) of 293T cells were incubated with or without 10 μg/ml NSF, 5 μg/ml α-SNAP and 0.5 mM ATP in the presence or absence of 8 mM $MgCl_2$ at 16°C for 60 min. After incubation, the samples were immunoprecipitated with a mAb to syntaxin 18 (clone 1E1) (lanes 1–4) or a polyclonal Ab to p31 (lanes 5–8), RINT-1 (lanes 9–12) or ZW10 (lanes 13–16). The precipitated proteins were separated and analyzed by immunoblotting with the indicated Abs. (**E**) Samples were prepared, treated and analyzed as described in (D). Immunoprecipitation with a polyclonal Ab to ZW10 (lanes 1 and 2) or a mAb to syntaxin 18 (clone 1E1) (lanes 3 and 4). We also analyzed 4% of the lysate (lane 5). Asterisks (bottom strip) denote rabbit immunoglobulin light chain.

suggests that p31 is a mammalian ortholog of Use1p/Slt1p (Dilcher *et al*, 2003). RINT-1 is a Rad50-interacting protein that participates in radiation-induced G_2/M checkpoint control (Xiao *et al*, 2001), and ZW10 is a kinetochore-binding protein involved in the spindle checkpoint (Williams *et al*, 1992, 1996; Williams and Goldberg, 1994; Starr *et al*, 1997, 1998). The roles of RINT-1 and ZW10 during interphase are not known.

To substantiate the specificity of the binding of p31, RINT-1 and ZW10 to syntaxin 18, we raised polyclonal Abs that are capable of specifically recognizing each antigen (Figure 1B). First, we examined whether these three proteins are associated not only with syntaxin 18 but also with syntaxin 5, a Golgi-located syntaxin (Dascher *et al*, 1994). As shown in Figure 1C, none of them were immunoprecipitated with a polyclonal anti-syntaxin 5 Ab. Next, we used another anti-syntaxin 18 mAb (clone 1E1) for immunoprecipitation. Epitope mapping analysis revealed that mAbs 2A6 and 1E1 recognize amino acids 223–229 and a sequence within amino acids 104–222, respectively (data not shown). As shown in Figure 1D, lane 1, p31, RINT-1 and ZW10 were co-precipitated with mAb 1E1. In a reciprocal experiment, syntaxin 18 was co-precipitated with the anti-p31 Ab (lane 5). It should be noted that not only syntaxin 18 but also RINT-1 and ZW10 were co-precipitated. Similar precipitation results were obtained with the Abs to RINT-1 (lane 9) and ZW10 (lane 13), although the efficiency of co-precipitation of syntaxin 18 with the latter Ab was somewhat low.

As a first step to gain insight into protein–protein interactions between the components of the syntaxin 18 complex, we performed two-hybrid assays. The results of β-galactosidase filter assay are summarized in Table I. Consistent with

Table I Two-hybrid interaction between proteins in the syntaxin 18 complex

GAL4 DNA-binding domain	GAL 4 activation domain	β-Galactosidase activity
α-SNAP	p31	+
α-SNAP	RINT-1	–
α-SNAP	ZW10	–
Syntaxin 18	p31	+
Syntaxin 18	RINT-1	–
Syntaxin 18	ZW10	–
ZW10	RINT-1	+
ZW10	P31	–
RINT-1	P31	–

To detect β-galactosidase activity, filters were incubated at 30°C for up to 3 h.

the fact that p31 contains a SNARE motif, it can interact directly with α-SNAP and syntaxin 18. RINT-1 and ZW10 interact with each other, whereas neither of them interacts with α-SNAP, syntaxin 18 or p31.

Disassembly of the syntaxin 18 complex

Conventional SNAREs are known to form a complex with NSF via α-SNAP, and the complex is disassembled upon ATP hydrolysis catalyzed by NSF (Söllner *et al*, 1993). We next examined if p31, RINT-1 and ZW10 behave similarly to conventional SNAREs. Upon incubation of 293T lysates with Mg^{2+}-ATP in the presence of NSF and α-SNAP, all three components dissociated from syntaxin 18 (Figure 1D, lane 2). This dissociation required both NSF and α-SNAP (lane 3) and Mg^{2+} (lane 4). Essentially the same results were obtained in immunoprecipitation experiments involving the Abs to p31 (lanes 6–8), RINT-1 (lanes 10–12) and ZW10 (lanes 14–16). Interestingly, p31, RINT-1 and ZW10 appeared to be still associated with one another even after they had dissociated from syntaxin 18 upon Mg^{2+}-ATP treatment (lanes 6, 10 and 14). These results suggest that p31, RINT-1 and ZW10 form a subcomplex in the syntaxin 18 complex. It should be noted that Sly1p or Sec22b was not co-precipitated with the anti-ZW10 Ab even under conditions that do not cause SNARE complex disassembly (Figure 1E). This suggests that the subcomplex does not contain Sly1p or Sec22b.

ZW10 is present in the ER and cytosol during interphase

Our finding that ZW10 is a component of the syntaxin 18 complex is quite surprising because ZW10 is known to be a component of the kinetochores and plays an important role in termination of the spindle checkpoint (Williams *et al*, 1992, 1996; Williams and Goldberg, 1994; Starr *et al*, 1997, 1998). Since the localization of ZW10 during interphase had not been precisely investigated, we next examined, by immunofluorescence microscopy, whether ZW10 is really located in the ER during interphase (Figure 2A). The overall immunostaining pattern of ZW10 was similar to that of an ER marker, calnexin (top and second rows). However, the patterns did not overlap completely, especially in the cell periphery. This may imply that ZW10 is restricted to distinct ER subdomains or present on additional intracellular compartments. In agreement with the binding result, ZW10 was found to be extensively colocalized with syntaxin 18 (third row). Our anti-ZW10 Ab stained $p150^{Glued}$-positive kinetochores and spindle poles in mitotic cells (fourth row), ruling out the possibility that it predominantly recognized protein(s) different from ZW10 on immunofluorescence analysis. As an alternative approach to determining the localization of ZW10, we produced a HeLa cell line in which a ZW10–green fluorescent protein (GFP) chimera is inducible by doxycycline. When the expression of the chimera was induced, the expressed protein exhibited a reticular pattern, which overlapped significantly with the staining profile of Hsp47, an ER lumenal protein (fifth row). Although ZW10–GFP was overexpressed at certain levels, it was still targeted to $p150^{Glued}$-positive kinetochores and spindle poles (bottom row), implying the functional integrity of the expressed ZW10–GFP.

As ZW10 interacts with dynamitin (Starr *et al*, 1998), a subunit of the dynactin complex involved in microtubule-dependent transport (Echeverri *et al*, 1996), we wondered if ZW10 is present on microtubules. As shown in Figure 2B, although there was some overlap in the localization of ZW10 and α-tubulin, ZW10 was also present in areas devoid of α-tubulin. In addition, nocodazole treatment did not markedly change the distribution patterns of ZW10 and ZW10–GFP.

To corroborate the ER localization of ZW10, we performed subcellular fractionation with OptiPrep (Figure 2C). ER-derived membrane vesicles (fractions 9–17) were substantially separated from Golgi vesicles (fractions 3–7) and endosomes (fractions 1–5). Obviously, ZW10, RINT-1 and p31, as well as syntaxin 18, were observed in fractions corresponding to the ER ones. α-Tubulin was not detected in the fractions comprising ZW10. The amounts of ZW10 and RINT-1 in the membrane fraction were comparable to those in the cytosol (Figure 2C, left panel), although they do not have transmembrane domains. p31 was found to be associated almost exclusively with ER membranes. These results combined with those of immunofluorescence analysis suggest that a significant fraction of ZW10 is associated with ER membranes during interphase.

Overexpression of ZW10 perturbs trafficking between the ER and Golgi

Our previous study demonstrated that syntaxin 18 plays roles in ER morphology and protein transport between the ER and Golgi (Hatsuzawa *et al*, 2000). Therefore, the most intriguing question is if ZW10 participates in membrane trafficking during interphase. To address this question, we first examined the effect of overexpression of ZW10 on the morphology of organelles within the early secretory pathways. As shown in Figure 3A, overexpression of ZW10 caused dispersion of a tethering protein in the *cis*-Golgi, p115 (Waters *et al*, 1992), an integral membrane protein in the ER–Golgi intermediate compartment, ERGIC-53 (Schweizer *et al*, 1990), a *cis*-Golgi integral membrane protein, syntaxin 5 (Dascher *et al*, 1994) and a *cis*-Golgi matrix protein, GM130 (Nakamura *et al*, 1995). Sec31p, a component of the COPII coat involved in export from the ER, was observed throughout the peripheral cytoplasm but concentrated in the perinuclear region in intact cells (Tang *et al*, 2000). Upon overexpression of ZW10, the intensity of the Sec31p staining in the perinuclear region became weak. On the other hand, no significant change was observed in the morphology of an ER protein, calnexin (Figure 3A), or microtubules (data not shown). These results suggest that membrane trafficking within the early secretory pathways is perturbed by overexpression of ZW10.

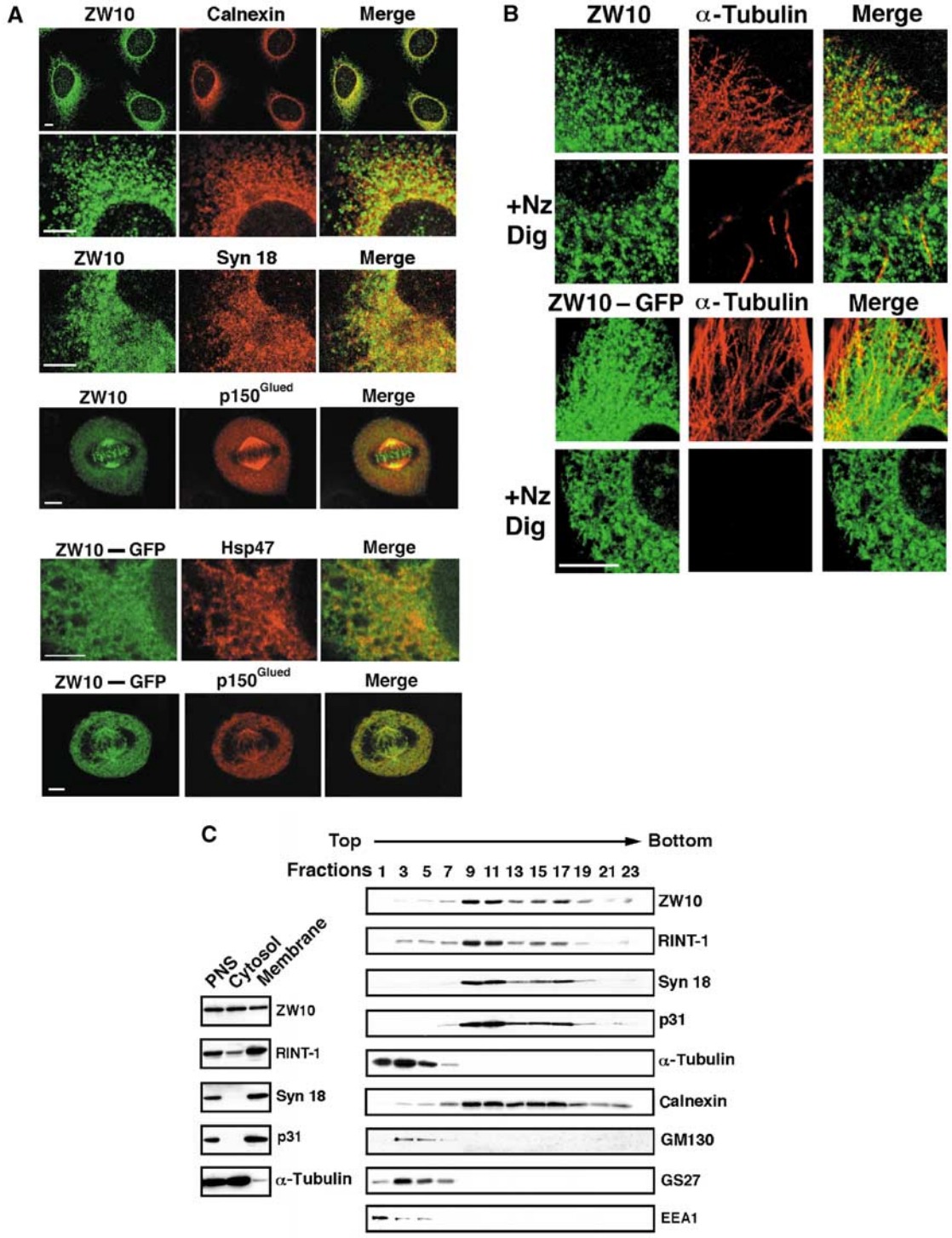

Figure 2 ZW10 is present in the ER during interphase. (**A**) HeLa cells were permeabilized with 30 μg/ml digitonin and double stained with Abs to ZW10 and calnexin (top and second rows), ZW10 and syntaxin 18 (third row) or ZW10 and dynactin p150^{Glued} (fourth row). In addition, HeLa cells expressing ZW10–GFP were permeabilized and stained with an Ab to Hsp47 (fifth row) or p150^{Glued} (bottom row). Bars, 5 μm. (**B**) HeLa cells were treated without (first row) or with 10 μg/ml nocodazole for 2 h (second row), and double stained with Abs to ZW10 and α-tubulin. Alternatively, ZW10–GFP-expressing cells were treated without (third row) or with nocodazole (bottom row), and stained with an Ab to α-tubulin. In the case of the cells treated with nocodazole, depolymerized tubulin was removed by digitonin treatment before fixation. (**C**) The postnuclear supernatant (4.2 mg) of HeLa cell extracts was separated into the cytosol (1.84 mg) and membrane fraction (1.67 mg). Portions of the fractions (20 μg each) were analyzed by immunoblotting with the indicated Abs. The membrane fraction was further fractionated as described in Materials and methods. Every other fraction was analyzed by immunoblotting with the indicated antibodies. The following proteins were used as organelle markers: Calnexin (ER), GM130 and GS27 (Golgi) and EEA1 (early endosomes).

To explore the possibility that anterograde transport from the ER is inhibited by ZW10 overexpression, we measured the transport of VSVG–GFP, a chimera protein comprising GFP and vesicular stomatitis virus-encoded glycoprotein (VSVG), whose export from the ER occurs in a temperature-dependent manner (Presley *et al*, 1997). As shown in Figure 3B (top

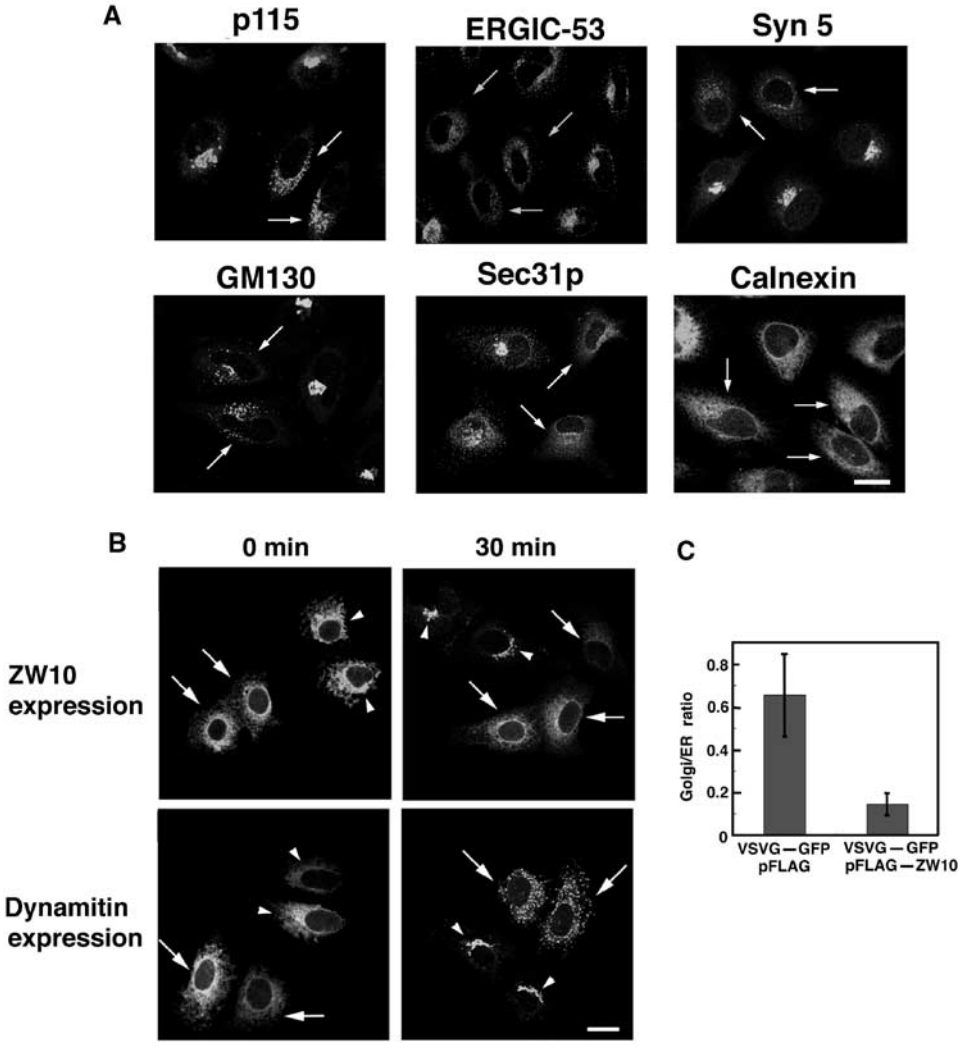

Figure 3 Effects of ZW10 overexpression on membrane trafficking between the ER and Golgi. (**A**) HeLa cells were transfected with the plasmid for FLAG–ZW10. After 24 h, the cells were fixed and double stained with Abs to FLAG (data not shown) and p115, ERGIC-53, syntaxin 5, GM130, Sec31p or calnexin. Arrows indicate cells overexpressing FLAG–ZW10. Bar, 20 μm. (**B**) The plasmid for VSVG–GFP was co-transfected with the plasmid for FLAG–ZW10 or FLAG–dynamitin into Vero cells, and the cells were incubated at 40°C for 24 h. The cells were then shifted to 32°C and incubated for 30 min. Before or after the temperature shift, the cells were fixed. To identify cells expressing FLAG–ZW10 or FLAG–dynamitin, the fixed cells were stained with an anti-FLAG Ab followed by a Texas Red-conjugated secondary Ab. Only GFP fluorescence is shown. Arrows and arrowheads indicate cells overexpressing FLAG-tagged proteins and nonexpressing cells, respectively. Bar, 20 μm. (**C**) Quantitation of the Endo H-resistant Golgi form versus the Endo H-sensitive ER form of VSVG–GFP.

right), at 30 min after temperature shift to 32°C (permissive temperature), VSVG–GFP remained in the ER in ZW10-overexpressing cells (arrows), whereas it reached the perinuclear Golgi area in control cells (arrow heads).

To provide biochemical evidence for the blockage of VSVG–GFP transport to the Golgi in ZW10-overexpressing cells, we investigated the endoglycosidase H (Endo H) sensitivity of VSVG–GFP. When VSVG protein is transported from the ER to the Golgi, it acquires Endo H resistance. At 60 min after temperature shift to 32°C, a substantial amount of VSVG–GFP acquired Endo H resistance in control cells, whereas only a small fraction of VSVG–GFP showed Endo H resistance in cells transfected with the plasmid for FLAG–ZW10 (data not shown). The ratio of the Endo H-resistant Golgi form versus the Endo H-sensitive ER form was 0.16 in cells transfected with the plasmid for FLAG–ZW10 compared to 0.65 in control cells (Figure 3C).

Overexpression of ZW10 does not disrupt the dynein–dynactin complex

Given that ZW10 interacts with dynamitin (Starr *et al*, 1998), a subunit of the dynactin complex (Echeverri *et al*, 1996), one explanation for the ZW10-induced defect in membrane trafficking between the ER and Golgi is that overexpression of ZW10, like that of dynamitin (Burkhardt *et al*, 1997), impairs the dynein–dynactin function by disrupting the dynactin complex. However, several lines of evidence excluded this possibility. First, VSVG–GFP was distributed uniformly in the entire ER in ZW10-expressing cells at 30 min after the temperature shift (Figure 3B, top right), whereas it was concentrated in dot-like structures, which represent dilated ER exit sites (Presley *et al*, 1997; Storrie *et al*, 1998), in dynamitin-overexpressing cells (bottom right). These results suggest that the steps inhibited by overexpression of ZW10 and dynamitin are different from each other. Second, although

overexpression of dynamitin, as reported previously (Burkhardt *et al*, 1997), substantially blocked dynein-mediated movement of incorporated FITC–transferrin toward the cell center, ZW10 overexpression had no marked effect (Figure 4A). Third, overexpression of dynamitin caused $p150^{Glued}$, a subunit of the dynactin complex (Paschal *et al*, 1993), to be redistributed from short linear array-like structures to fuzzy, disintegrated ones, but overexpression of ZW10 did not (Figure 4B). Lastly and importantly, sedimentation analysis revealed that overexpression of ZW10, unlike that of dynamitin, did not disrupt the dynactin complex (Figure 4C, middle).

Association of ZW10 with RINT-1 and syntaxin 18 is required for disruption of the Golgi

To assess the specificity of the effect of overexpression of ZW10, the N-terminal region (amino acids 1–316), central region (amino acids 257–537) or C-terminal region (amino acids 468–779) of ZW10 was expressed, and the localization of p115 and Sec31p was investigated. As shown in Figure 5A, the N-terminal fragment but not the central or C-terminal one induced redistribution of p115 and Sec31p. Immunoprecipitation experiments demonstrated that RINT-1 and syntaxin 18 were co-precipitated with the N-terminal fragment (Figure 5B, lane 7) but not with the central (lane 8) or C-terminal one (lane 9). This correlation strongly suggests that overexpressed ZW10 disrupts membrane trafficking in the early secretory pathway by interacting with the syntaxin 18 complex.

Loss of ZW10 function affects membrane trafficking between the ER and Golgi

To provide additional evidence for the involvement of ZW10 in membrane trafficking between the ER and Golgi, we microinjected an affinity-purified anti-ZW10 Ab into HeLa cells. As shown in Figure 6, the anti-ZW10 microinjection induced dispersion of ER–Golgi proteins such as p115, GM130 and ERGIC-53. Microinjection of control rabbit IgG, on the other hand, had no significant effect on the distributions of these proteins.

To investigate the requirement of ZW10 activity for membrane trafficking, we employed RNA interference with 21-base RNA duplexes (Elbashir *et al*, 2001). We used two different RNA duplexes, ZW10(102) and ZW10(1911). As shown in Figure 7A, the level of expression of ZW10 was

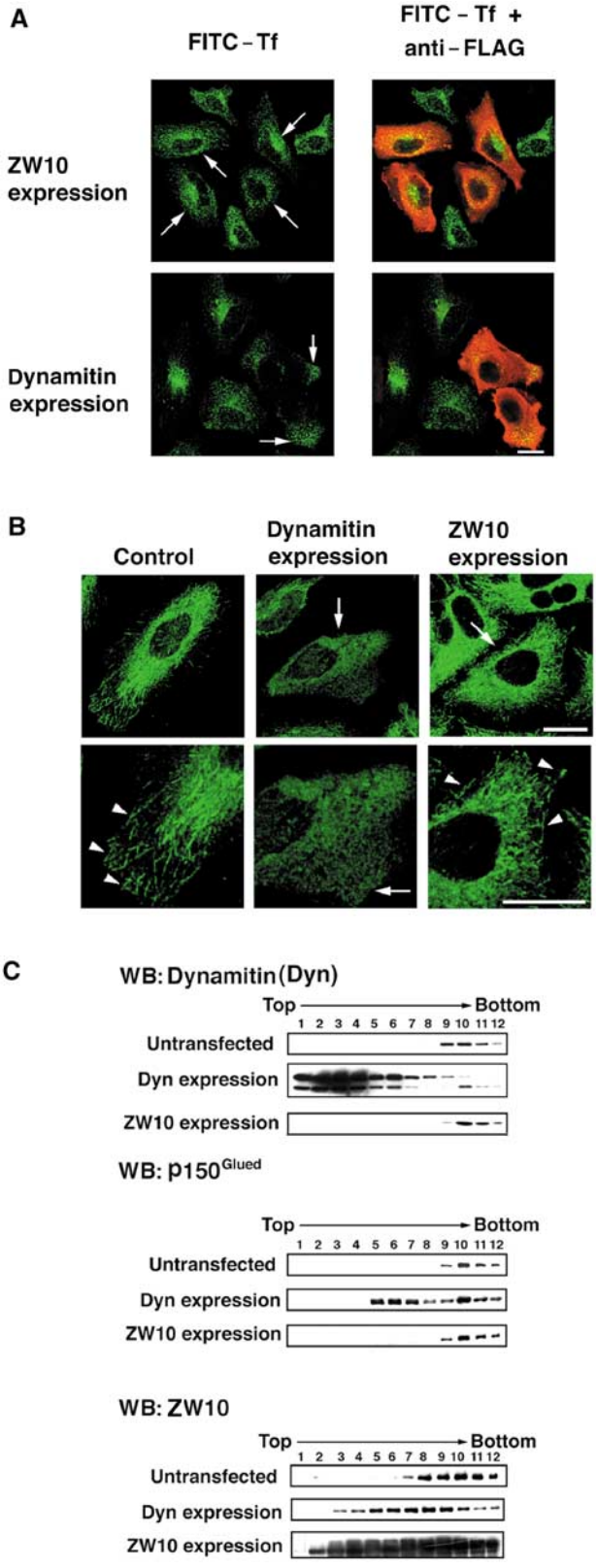

Figure 4 Integrity of the dynactin complex in cells overexpressing ZW10. (**A**) FLAG–ZW10 or FLAG–dynamitin was overexpressed in HeLa cells, and FITC–transferrin uptake experiments were conducted as described in Materials and methods. The cells were fixed and stained with an anti-FLAG Ab followed by a Texas Red-conjugated secondary Ab. FITC fluorescence and both FITC and Texas Red fluorescences are shown. Arrows indicate ZW10- or dynamitin-overexpressing cells. Bar, 10 μm. (**B**) HeLa cells were transfected with the plasmid for FLAG–dynamitin or FLAG–ZW10. After 24 h, the cells were fixed and double stained with Abs to $p150^{Glued}$ (FITC) and FLAG (Texas Red; not shown). Arrows indicate dynamitin- or ZW10-overexpressing cells. Arrowheads denote the presence of $p150^{Glued}$ in short linear arrays in the cell periphery. Images at a low magnification (top) and a high magnification (bottom) are shown. Bars, 10 μm. (**C**) 293T cells were untransfected or transfected with the plasmid for FLAG–dynamitin or FLAG–ZW10. After 24 h, cell lysates were prepared and fractionated by sedimentation. Fractions were recovered from the top and analyzed by Western blotting (WB) using Abs to dynamitin (top), $p150^{Glued}$ (middle) and ZW10 (bottom). Note that $p150^{Glued}$ showed a shift in sedimentation profile in cells overexpressing dynamitin but not ZW10 (middle).

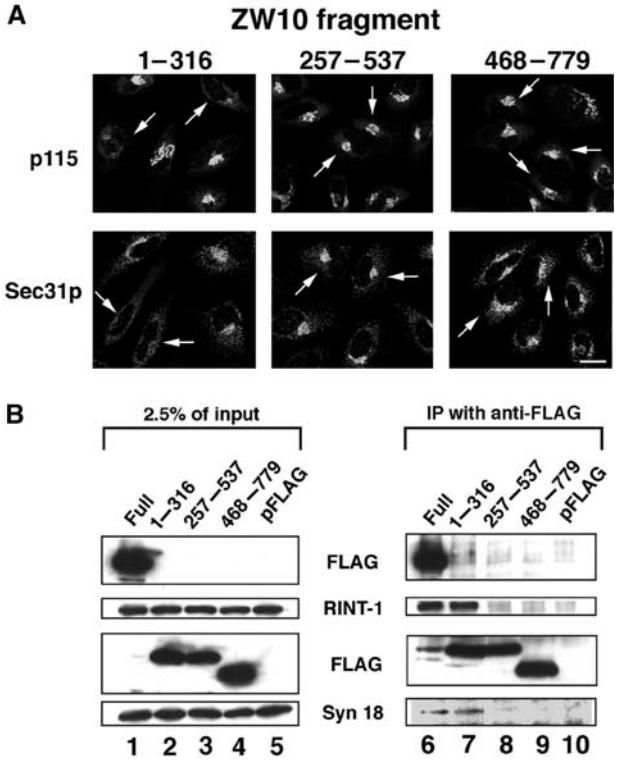

Figure 5 Association of ZW10 with syntaxin 18 is required for disruption of the Golgi. (**A**) HeLa cells were transfected with the plasmid encoding ZW10 amino acids 1–316, amino acids 257–537 or amino acids 468–779, each with FLAG tag. After 24 h, the cells were fixed and double stained with Abs to FLAG and p115 or Sec31p. Arrows indicate cells overexpressing the fragments. Bar, 20 μm. (**B**) 293T cells grown in a 35 mm dish were transfected with the plasmids encoding the indicated constructs with FLAG tag. At 24 h after transfection, cell lysates were prepared and FLAG-tagged proteins were immunoprecipitated with an anti-FLAG Ab. The immunoprecipitated proteins were analyzed by immunoblotting with Abs to RINT-1, syntaxin 18 and FLAG (lanes 6–10). Lanes 1–5 show the amounts of RINT-1, syntaxin 18 and FLAG-tagged proteins in 2.5% of the lysate.

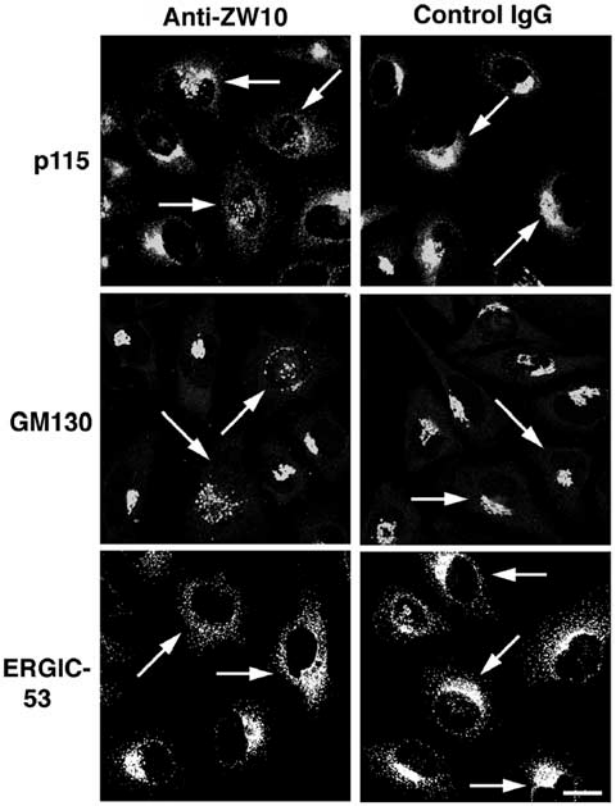

Figure 6 Microinjection of an anti-ZW10 Ab causes dispersion of compartments between the ER and Golgi. At 20 h after microinjection of an affinity-purified anti-ZW10 Ab or control IgG into HeLa cells, the cells were fixed and stained with Abs to p115, GM130 and ERGIC-53. To identify cells injected with the Ab, the injected Ab was visualized by staining with a Texas Red-conjugated anti-rabbit IgG Ab (not shown). Arrows indicate antibody-injected cells.

reduced markedly by ZW10(102) but only slightly by ZW10(1911). We observed that cell growth was not arrested in cells in which ZW10 expression was markedly suppressed, although the growth rate was somewhat lower compared to control cells. Perhaps, residual ZW10 might afford to drive cell cycle progression. Cell cycle was not arrested when the expression levels of certain mitosis-related proteins were reduced by 80–90% compared with the original levels (T Urano, personal communication).

Concomitant with the reduction of ZW10 protein, the distribution of p115 significantly changed from a compact, perinuclear pattern to a dispersed one (Figure 7B, bottom). On the other hand, no marked change in the distribution of p115 was observed in cells transfected with ZW10(1911) or mock-treated cells, implying that the dispersion of p115 induced by ZW10(102) is not due to an artifact of transfection but a consequence of the reduction of ZW10 expression. Silencing of ZW10 affected markedly the distribution of p115, but less significantly those of other *cis*-Golgi proteins such as syntaxin 5 and GM130.

Electron microscopic analysis of cells with reduced levels of ZW10 expression showed the presence of mini-Golgi stacks (Figure 7C). Quantitative analysis confirmed that the length of the Golgi cisternae in cells with reduced levels of ZW10 is significantly shorter than that in mock-treated cells (Figure 7D). In addition, electron microscopic analysis showed that organelles other than the Golgi apparatus are normal in cells transfected with ZW10(102). No condensation of chromosomes in the nucleus was observed, in agreement with the idea that cell cycle is not arrested at G_2/M transition or M phase.

To assess the effect of ZW10 depletion on vesicular transport from the ER, we carried out a morphological VSVG–GFP transport assay (Figure 8). At 30 min after shift to the permissive temperature, VSVG–GFP was detected in the ERGIC and/or ER exit sites in cells with reduced levels of ZW10 expression, whereas it had been transported to the Golgi apparatus in mock-treated cells and cells transfected with ZW10(1911). In ZW10-depleted cells, VSVG–GFP had reached the plasma membrane by 120 min. These results suggest that depletion of ZW10 partially inhibits VSVG–GFP transport from the ER.

Discussion

The present results demonstrated that syntaxin 18 forms large complexes comprising conventional syntaxin-binding proteins (rSec22b, α-SNAP and rSly1p) and nonconventional

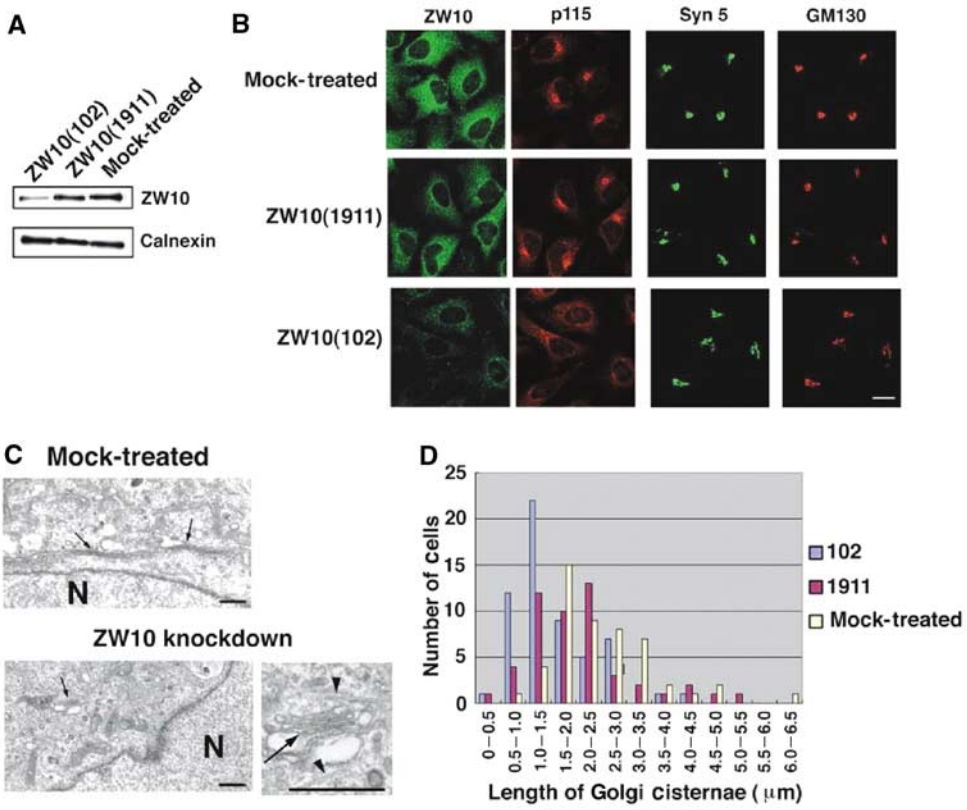

Figure 7 Silencing of ZW10 causes dispersion of p115. (**A**) HeLa cells were transfected without (mock-treated) or with RNA duplexes (ZW10(102) or ZW10(1911)). At 72 h after transfection, the cells were solubilized in phosphate-buffered saline with 0.5% SDS. Equal amounts of total proteins were separated by SDS–PAGE and analyzed by immunoblotting with Abs to ZW10 and, as a loading control, calnexin. (**B**) Immunofluorescence microscopic analysis of mock-treated cells, and cells transfected with ZW10(1911) or ZW10(102). At 72 h after transfection, the cells were double stained with Abs to ZW10 and p115 or syntaxin 5 and GM130. Bar, 20 μm. (**C**) Electron microscopic analysis of mock-treated cells and cells with reduced levels of ZW10. Arrows and arrowheads denote the Golgi apparatus and microtubules, respectively. N represents the nucleus. Bars, 500 nm. (**D**) Quantitation of the length of the Golgi cisternae. About 50 cells were counted in each experiment.

ones (ZW10, RINT-1 and p31). It is not certain whether SNAP-23 is really associated with syntaxin 18 because it was not immunoprecipitated with a mAb to syntaxin 18 (clone 1E1) or a polyclonal one (data not shown). Similar to the fact that yeast Sly1p interacts with syntaxins located in the Golgi (Sed5p) (Lupashin and Waters, 1997) and ER (Ufe1p) (Reilly *et al*, 2001; Yamaguchi *et al*, 2002), rSly1p binds to the mammalian counterpart of Sed5p, syntaxin 5 (Dascher and Balch, 1996), and the putative mammalian ortholog of Ufe1p, syntaxin 18.

Unexpectedly, ZW10, a spindle checkpoint protein, and RINT-1, a G_2/M checkpoint protein, were found to be associated with syntaxin 18. This finding is quite surprising, but several lines of evidence strongly suggest the association of syntaxin 18 with ZW10, RINT-1 and p31. First, the three proteins were co-precipitated with two different mAbs and a polyclonal Ab to syntaxin 18, but not with an anti-syntaxin 5 Ab. Second, syntaxin 18 was co-precipitated with Abs to any of the three proteins, although the efficiency of co-precipitation of syntaxin 18 with the anti-ZW10 Ab was somewhat low. Third, the three proteins dissociated from syntaxin 18 in an NSF, α-SNAP, Mg^{2+}-ATP-dependent manner, implying their functional linkage with syntaxin 18. Lastly, syntaxin 18 and RINT-1 were co-precipitated with expressed FLAG-tagged ZW10 and its N-terminal fragment with an anti-FLAG Ab.

Ufe1p, the putative yeast ortholog of syntaxin 18, is known to form a complex with Sec22p, Sec20p and Tip20p (Lewis *et al*, 1997). Sequence comparison revealed that RINT-1 exhibits low but significant sequence similarity to Tip20p (21% identity in amino acids 300–620 of RINT-1). Among mammalian proteins known at present, RINT-1 is the most similar to Tip20p. Ito *et al* (2001) showed that, in a yeast two-hybrid assay, Tip20p interacts with Dsl1p, a protein involved in retrograde transport from the Golgi to the ER (Andag *et al*, 2001; Reilly *et al*, 2001). Although RINT-1 interacts with ZW10 in a two-hybrid assay, no apparent sequence similarity was observed between Dsl1p and ZW10. We could not find a putative ortholog of Sec20p among the identified components of the syntaxin 18 complex.

Since we did not detect a protein corresponding to Rad50, a partner of RINT-1 (Xiao *et al*, 2001), in the syntaxin 18 complex, RINT-1 may exist in two distinct complexes, one involved in the G_2/M checkpoint and the other in membrane trafficking. This may also be the case for ZW10. ZW10 forms a complex with ROD (Scaerou *et al*, 2001), and the complex plays a role in turning off of the spindle checkpoint (Howell *et al*, 2001; Wojcik *et al*, 2001). The present study demonstrated that ROD is not present in the isolated syntaxin 18 complex.

How are the formation and activity of complexes comprising ZW10 and/or RINT-1 regulated? We can envisage two

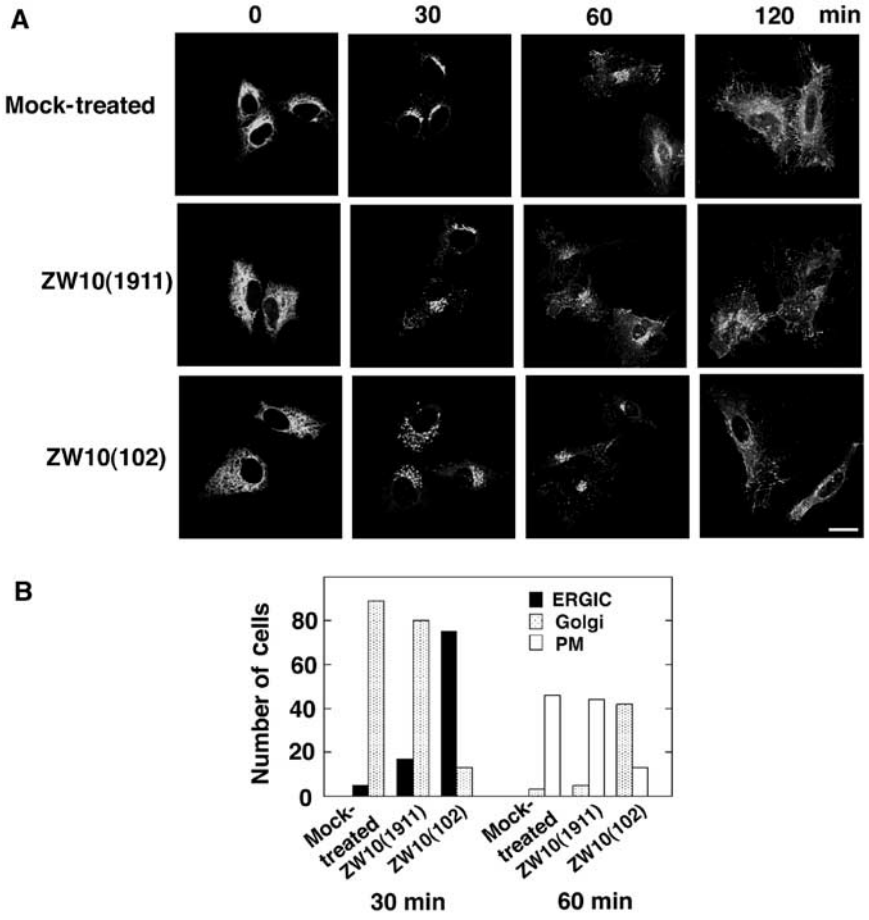

Figure 8 Delay in VSVG–GFP transport from the ER in cells with reduced levels of ZW10. HeLa cells were successively transfected with duplex RNA and the plasmid for VSVG–GFP. Transport of VSVG–GFP from the ER was monitored as described in Materials and methods. (**A**) Typical images at 0, 30, 60 and 120 min after temperature shift to 32°C are shown. (**B**) Quantitation of protein transport. The numbers of cells in which VSVG–GFP was located at the ERGIC and/or ER exit sites (ERGIC), Golgi (Golgi) and plasma membrane (PM) were individually counted. In all, 50–100 cells were analyzed.

plausible models. One model predicts that all complexes (ZW10–ROD, Rad50–RINT-1, ZW10–RINT-1–p31 and possibly others) are consistently present in cells and function independently. In this case, the ZW10–ROD and Rad50–RINT-1 complexes must be activated by some mechanisms at the onset of mitosis and G_2/M phase, respectively. The alternate model is that ZW10 and RINT-1 are mainly complexed with syntaxin 18 during interphase and change their partners depending on the cell cycle. This model comes from the finding that RINT-1 forms a complex with Rad50 only during G_2/M phase (Xiao *et al*, 2001). However, our preliminary result showed that the binding of ZW10 and RINT-1 to syntaxin 18 is independent of cell cycle (data not shown), making the latter model unlikely. Further studies are necessary to address this question.

Overexpression of ZW10 and microinjection of an anti-ZW10 Ab markedly disrupted membrane trafficking between the ER and Golgi. On the other hand, a reduction in ZW10 expression partially inhibited VSVG–GFP transport from the ER and did not severely disrupt the Golgi apparatus. Perhaps, residual ZW10 could manage vesicular transport, albeit with a low efficiency. It is possible that ZW10 with respect to its function and/or interaction is intimately related to p115, but less to syntaxin 5 and GM130. Therefore, depletion of ZW10 would have a profound effect on the distribution of p115, but not on those of syntaxin 5 and GM130. These results are reminiscent of those of p115. Although microinjection of an anti-p115 Ab causes Golgi disassembly in mammalian cells (Alvarez *et al*, 1999; Puthenveedu and Linstedt, 2001), depletion of p115 in *Drosophila*, albeit disturbing the organization of ER exit sites, does not markedly affect protein transport from the ER (Kondylis and Rabouille, 2003).

The present results may shed light on possible crosstalk between membrane trafficking and the cell cycle checkpoint. In this context, the finding of Malhotra and colleagues (Sutterlin *et al*, 2002) that the Golgi apparatus may act as a sensor for controlling entry into mitosis in mammalian cells is quite interesting. They suggested the presence of a set of components that check the status of the Golgi apparatus and determine the cell cycle progression into mitosis. ER components such as ZW10 and RINT-1 may cooperate for entry into mitosis. It should be noted that ZW10 in *Drosophila* embryos is excluded from the nucleus during interphase and migrates into the nuclear volume at prometaphase (Williams and Goldberg, 1994). At the onset of mitosis, some components of the ER may migrate into the nuclear volume, and the Golgi and nuclear membrane components may be redistributed into the ER (Zaal *et al*, 1999; Daigle *et al*, 2001).

In the course of this study, a novel SNAP-25-interacting protein (SIP30) was discovered (Lee *et al*, 2002). SIP30 is the same protein as HZwint-1, a kinetochore component that interacts with ZW10 (Starr *et al*, 2000). The fact that HZwint-1/SIP30 interacts with SNAP-25 supports our view that ZW10 is involved in membrane trafficking through interaction with the SNARE complex.

Materials and methods

Abs

Purified bacterially expressed His-tagged syntaxin 18 lacking the transmembrane domain was mixed with Immun Easy Mouse Adjuvant (QIAGEN) and then injected into BALB/c mice. Hybridoma cells producing Abs were obtained according to the standard protocol. mAb 2A6 was crosslinked to protein G–sepharose using dimethyl pimelimidate. Polyclonal Abs to human p31, RINT-1 and ZW10 were raised to bacterially expressed His-tagged p31 (amino acids 1–231), RINT-1 (amino acids 1–531) and ZW10 (amino acids 93–779). The produced Abs were affinity purified using antigen-coupled beads. A mAb to ERGIC-53 was a generous gift from Dr H-P Hauri (University of Basel). A polyclonal Ab to rSly1p was prepared as described (Matsuo *et al*, 1997) and kindly supplied by Dr T Katayama and Dr Y Bando (Osaka University). Polyclonal Abs to human Sec31p and Sec22b were prepared in this laboratory. mAbs to p115, calnexin, dynamitin, EEA1, GS27/membrin and $p150^{Glued}$ were obtained from Transduction Laboratories. A mAb to Hsp47 was purchased from StressGen. Monoclonal and polyclonal Abs to FLAG were from Sigma Chemicals.

Identification of syntaxin 18 complexes

Livers from Wistar rats that had fasted overnight were removed and rinsed once in homogenization buffer (20 mM HEPES, pH 7.2, 150 mM KCl, 2 mM EDTA, 0.5 μg/ml leupeptin, 2 μM pepstatin, 2 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride and 1 mM dithiothreitol). The following procedures were carried out at 0–4°C. The livers were perfused with homogenization buffer, cut into small pieces and then homogenized in a Dounce homogenizer (five strokes). The homogenate was centrifuged at 600 *g* for 10 min to remove debris and nuclei. The supernatant was centrifuged at 10 000 *g* for 10 min and then at 100 000 *g* for 60 min to obtain total membranes. The membrane pellet was solubilized in homogenization buffer with 1% Triton X-100. The Triton X-100 extracts (240 mg) were incubated with sepharose beads bearing an anti-Syn 18 mAb (clone 2A6). After washing, the bound proteins were eluted with 0.1 M glycine.

The eluted proteins were concentrated, resolved by SDS–PAGE on a 7–15% gradient gel and then visualized with Coomassie blue. Each band was excised and subjected to in-gel proteolysis with lysyl endopeptidase. The digests were fractionated by HPLC and analyzed by microsequencing and mass spectrometry as described (Takeda *et al*, 2001).

Preparation of extracts of 293T cells

To prepare 293T cell lysates for immunoprecipitation, approximately 80% confluent cells grown on two 15 cm dishes were lysed in 8 ml of lysis buffer (20 mM HEPES, pH 7.2, 150 mM KCl, 2 mM EDTA, 0.5 μg/ml leupeptin, 2 μM pepstatin, 2 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol and 1% Triton X-100). The lysates were centrifuged in a microcentrifuge at 15 000 rpm for 10 min.

Establishment of Tet-on inducible ZW10–GFP expression cells

HeLa Tet-on cells (CLONTECH) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% Tet system approved fetal bovine serum (CLONTECH) and 200 μg/ml G418.

To establish stable transfectants, HeLa Tet-on cells were co-transfected with pTK–Hyg and pTRE–Tet–ZW10–EGFP. Drug-resistant clones were screened in the presence of 300 μg/ml hygromycin B. Expression of ZW10–GFP was induced by 1.0 μg/ml doxycycline.

Plasmid construction and transfection

The full-length cDNAs of p31, RINT-1, dynamitin and ZW10 were amplified by polymerase chain reaction using a human leukocyte cDNA library. Mammalian expression plasmid pFLAG–CMV-2 (Eastman Kodak Corp.) was used to express proteins with the N-terminal FLAG epitope. The cDNA fragments encoding the full-length ZW10 (amino acids 1–779), ZW10 (amino acids 1–316), ZW10 (amino acids 257–537), ZW10 (amino acids 468–779) and the full-length dynamitin were inserted into pFLAG–CMV-2. For RINT-1 binding assays, 293T cells were transfected with the expression plasmids (1 μg each) for ZW10 constructs using LipofectAMINE PLUS reagent according to the manufacturer's protocol. Immunoprecipitation experiments were carried out as described (Hatsuzawa *et al*, 2000).

To construct the plasmid encoding ZW10 fused to the N-terminus of GFP, the full-length cDNA of human ZW10 was subcloned into pEGFP-N1. The DNA fragment encoding ZW10–GFP was amplified by polymerase chain reaction, and the amplified fragment was inserted into pTRE vector.

Microinjection

Microinjection was conducted as described (Fukunaga *et al*, 1998). An affinity-purified anti-ZW10 Ab (10.0 mg/ml) or rabbit IgG (27.5 mg/ml) was injected with an injection time of 0.2 s.

Immunofluorescence and electron microscopy

Immunofluorescence microscopy was performed as described (Tagaya *et al*, 1996). Cells were fixed with methanol at −20°C for 5 min for endogenous ZW10 or with 4% paraformaldehyde for 20 min at room temperature for expressed proteins. Confocal microscopy was performed with an Olympus Fluoview 300 laser scanning microscope. FITC–transferrin uptake experiments were conducted as described (Fukunaga *et al*, 2000). Electron microscopy was performed as described (Yamaguchi *et al*, 1997).

Subcellular fractionation

Approximately 80% confluent HeLa cells (three 15 cm dishes) were washed twice in phosphate-buffered saline and then once in homogenization buffer (10 mM HEPES–KOH, pH 7.5, 0.25 M sucrose and 1 mM EDTA). The cells were collected, suspended in 1 ml of homogenization buffer and then homogenized with 20 strokes in a stainless-steel homogenizer. The homogenate was centrifuged at 1000 *g* for 10 min, and then the supernatant was centrifuged at 100 000 *g* for 30 min to separate the cytosol and membrane fraction. The membrane fraction was suspended and layered onto a 0–28% OptiPrep (NYCOMED) gradient and centrifuged at 200 000 *g* for 6 h. After centrifugation, 24 fractions (0.17 ml each) were collected from the top to bottom.

Sedimentation analysis

293T cells (one 60 mm dish) were transfected with the plasmid (4 μg) for FLAG–ZW10 or FLAG–dynamitin. After 24 h, the cells were lysed in 0.5 ml of lysis buffer, and the lysate was centrifuged at 3000 rpm for 10 min and then at 50 000 rpm (TLA100.3 rotor, Beckman) for 30 min to obtain the supernatant. The supernatant was placed on the top of a 12–48% glycerol gradient and centrifuged at 34 000 rpm for 16 h (SW50.1 rotor, Beckman). After centrifugation, 12 fractions (0.3 ml each) were collected from the top to bottom, and analyzed for the presence of dynamitin, $p150^{Glued}$ and ZW10 by immunoblotting.

Protein transport from the ER to the Golgi

The expression plasmid for VSVG–GFP was kindly donated by Dr J Lippincott-Schwartz (National Institutes of Health, USA). The plasmid (1 μg) was co-transfected with the plasmid (1 μg) for FLAG–ZW10 or FLAG–dynamitin into Vero cells. The cells were incubated at 40°C for 24 h and then shifted to 32°C to allow transport. The cells were fixed and processed for immunofluorescence analysis. In the case of the biochemical transport assay, the cells were harvested at 60 min after the temperature shift, lysed in denaturing buffer (0.15 ml/35 mm dish) containing 0.5% SDS and 1% 2-mercaptoethanol and heated at 100°C for 10 min. A portion of the lysate was digested with Endo H (NEW ENGLAND BioLabs) and then subjected to SDS–PAGE on an 8% gel followed by immunoblotting. The intensities of immunostained bands were quantified using the NIH image.

RNA interference

RNA duplexes used for targeting were ZW10(102) (AAGGGTGA GGTGTGCAATATG) and ZW10(1911) (AATTGTGTGGCAGGATGTCCT). The RNA duplexes were purchased from Japan Bioservice, Inc. Transfection of HeLa cells, which had been grown in six-well plates, was performed using Oligofectamine (Life Technologies) according to the manufacturer's protocol. The final concentration of RNA duplexes was 100 nM. At 72 h after transfection, the cells were processed for immunoblotting, immunofluorescence and electron microscopic analyses. In the case of double transfection, cells were first transfected with duplex RNAs, incubated for 24 h, and then transfected with the plasmid for VSVG–GFP. Transport of VSVG–GFP was monitored after further 48 h incubation.

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References

Alvarez C, Fujita H, Hubbard A, Sztul E (1999) ER to Golgi transport: requirement for p115 at a pre-Golgi VTC stage. *J Cell Biol* **147:** 1205–1211

Andag U, Neumann T, Schmitt HD (2001) The coatomer-interacting protein Dsl1p is required for Golgi-to-endoplasmic reticulum retrieval in yeast. *J Biol Chem* **276:** 39150–39160

Belgareh-Touze N, Corral-Debrinski M, Launhardt H, Galan JM, Munder T, Le Panse S, Haguenauer-Tsapis R (2003) Yeast functional analysis: identification of two essential genes involved in ER to Golgi trafficking. *Traffic* **4:** 607–617

Burkhardt JK, Echeverri CJ, Nilsson T, Vallee RB (1997) Overexpression of the dynamitin (p50) subunit of the dynactin complex disrupts dynein-dependent maintenance of membrane organelle distribution. *J Cell Biol* **139:** 469–484

Burri L, Varlamov O, Doege CA, Hofmann K, Beilharz T, Rothman JE, Söllner TH, Lithgow T (2003) A SNARE required for retrograde transport to the endoplasmic reticulum. *Proc Natl Acad Sci USA* **100:** 9873–9877

Chan GKT, Jablonski SA, Starr DA, Goldberg ML, Yen TJ (2000) Human Zw10 and ROD are mitotic checkpoint proteins that bind to kinetochores. *Nat Cell Biol* **2:** 944–947

Cleveland DW, Mao Y, Sullivan KF (2003) Centromeres and kinetochores: from epigenetics to mitotic checkpoint signaling. *Cell* **112:** 407–421

Daigle N, Beaudouin J, Hartnell L, Imreh G, Hallberg E, Lippincott-Schwartz J, Ellenberg J (2001) Nuclear pore complexes form immobile networks and have a very low turnover in live mammalian cells. *J Cell Biol* **154:** 71–84

Dascher C, Balch WE (1996) Mammalian Sly1 regulates syntaxin 5 function in endoplasmic reticulum to Golgi transport. *J Biol Chem* **271:** 15866–15869

Dascher C, Matteson J, Balch WE (1994) Syntaxin 5 regulates endoplasmic reticulum to Golgi transport. *J Biol Chem* **269:** 29363–29366

Dilcher M, Veith B, Chidambaram S, Hartmann E, Schmitt HD, Fischer von Mollard G (2003) Use1p is a yeast SNARE protein required for retrograde traffic to the ER. *EMBO J* **22:** 3664–3674

Echeverri CJ, Paschal BM, Vaughan KT, Vallee RB (1996) Molecular characterization of the 50-kD subunit of dynactin reveals function for the complex in chromosome alignment and spindle organization during mitosis. *J Cell Biol* **132:** 617–633

Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T (2001) Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* **411:** 494–498

Fukunaga T, Furuno A, Hatsuzawa K, Tani K, Yamamoto A, Tagaya M (1998) NSF is required for the brefeldin A-promoted disassembly of the Golgi apparatus. *FEBS Lett* **435:** 237–240

Fukunaga T, Nagahama M, Hatsuzawa K, Tani K, Yamamoto A, Tagaya M (2000) Implication of sphingolipid metabolism in the stability of the Golgi apparatus. *J Cell Sci* **113:** 3299–3307

Hatsuzawa K, Hirose H, Tani K, Yamamoto A, Scheller RH, Tagaya M (2000) Syntaxin 18, a SNAP receptor that functions in the endoplasmic reticulum, intermediate compartment, and *cis*-Golgi vesicle trafficking. *J Biol Chem* **275:** 13713–13720

Howell BJ, McEwen BF, Canman JC, Hoffman DB, Farrar EM, Rieder CL, Salmon ED (2001) Cytoplasmic dynein/dynactin drives kinetochore protein transport to the spindle poles and has a role in mitotic spindle checkpoint inactivation. *J Cell Biol* **155:** 1159–1172

Ito T, Chiba T, Ozawa R, Yoshida M, Hattori M, Sakaki Y (2001) A comprehensive two-hybrid analysis to explore the yeast protein interactome. *Proc Natl Acad Sci USA* **98:** 4569–4574

Jahn R, Lang T, Südhof TC (2003) Membrane fusion. *Cell* **112:** 519–533

Kondylis V, Rabouille C (2003) A novel role of dp115 in the organization of tER sites in *Drosophila*. *J Cell Biol* **162:** 185–198

Lee J-K, Safieddine S, Petralia RS, Wenthold RJ (2002) Identification of a novel SNAP25 interacting protein (SIP30). *J Neurochem* **81:** 1338–1347

Lewis M, Pelham HRB (1996) SNARE-mediated retrograde traffic from the Golgi complex to the endoplasmic reticulum. *Cell* **85:** 205–215

Lewis MJ, Rayner JC, Pelham HR (1997) A novel SNARE complex implicated in vesicle fusion with the endoplasmic reticulum. *EMBO J* **16:** 3017–3024

Lin RC, Scheller RH (2000) Mechanisms of synaptic vesicle exocytosis. *Annu Rev Cell Dev Biol* **16:** 19–49

Lupashin VV, Waters MG (1997) t-SNARE activation through transient interaction with a Rab-like guanosine triphosphatase. *Science* **276:** 1255–1258

Matsuo N, Ogawa S, Takagi T, Wanaka A, Mori T, Matsuyama T, Pinsky DJ, Stern DM, Tohyama M (1997) Cloning of a putative vesicle transport-related protein, RA410, from cultured rat astrocytes and its expression in ischemic rat brain. *J Biol Chem* **272:** 16438–16444

Millband DN, Campbell L, Hardwick KG (2002) The awesome power of multiple model systems: interpreting the complex nature of spindle checkpoint signaling. *Trends Cell Biol* **12:** 205–209

Nakamura N, Rabouille C, Watson R, Nilsson T, Hui N, Slusarewicz P, Kreis TE, Warren G (1995) Characterization of a *cis*-Golgi matrix protein, GM130. *J Cell Biol* **131:** 1715–1726

Paschal BM, Holzbaur EL, Pfister KK, Clark S, Meyer DI, Vallee RB (1993) Characterization of a 50-kDa polypeptide in cytoplasmic dynein preparations reveals a complex with $p150^{GLUED}$ and a novel actin. *J Biol Chem* **268:** 15318–15323

Patel SK, Indig FE, Olivieri N, Levine ND, Latterich M (1998) Organelle membrane fusion: a novel function for the syntaxin homolog Ufe1p in ER membrane fusion. *Cell* **92:** 611–620

Presley JF, Cole NB, Schroer TA, Hirschberg K, Zaal KJ, Lippincott-Schwartz J (1997) ER-to-Golgi transport visualized in living cells. *Nature* **389:** 81–85

Puthenveedu MA, Linstedt AD (2001) Evidence that Golgi structure depends on a p115 activity that is independent of the vesicle tether components giantin and GM130. *J Cell Biol* **155:** 227–237

Reilly BA, Kraynack BA, VanRheenen SM, Waters MG (2001) Golgi-to-endoplasmic reticulum (ER) retrograde traffic in yeast requires Dsl1p, a component of the ER target site that interacts with a COPI coat subunit. *Mol Biol Cell* **12:** 3783–3796

Savoian MS, Goldberg ML, Rieder CL (2000) The rate of poleward chromosome motion is attenuated in *Drosophila zw10* and *rod* mutants. *Nat Cell Biol* **2:** 948–953

Scaerou F, Starr DA, Piano F, Papoulas O, Karess RE, Goldberg ML (2001) The ZW10 and Rough Deal checkpoint proteins function together in a large, evolutionarily conserved complex targeted to the kinetochore. *J Cell Sci* **114:** 3103–3114

Schweizer A, Fransen JA, Matter K, Kreis TE, Ginsel L, Hauri H-P (1990) Identification of an intermediate compartment involved in protein transport from endoplasmic reticulum to Golgi apparatus. *Eur J Cell Biol* **53:** 185–196

Söllner T, Whiteheart SW, Brunner M, Erdjument-Bromage H, Geromanos S, Tempst P, Rothman JE (1993) SNAP receptors implicated in vesicle targeting and fusion. *Nature* **362:** 318–324

Starr DA, Saffery R, Li Z, Simpson AE, Choo KH, Yen TJ, Goldberg ML (2000) HZwint-1, a novel human kinetochore component that interacts with HZW10. *J Cell Sci* **113:** 1939–1950

Starr DA, Williams BC, Hays TS, Goldberg ML (1998) ZW10 helps recruit dynactin and dynein to the kinetochore. *J Cell Biol* **142:** 763–774

Starr DA, Williams BC, Li Z, Etemad-Moghadam B, Dawe RK, Goldberg ML (1997) Conservation of the centromere/kinetochore protein ZW10. *J Cell Biol* **138:** 1289–1301

Storrie B, White J, Rottger S, Stelzer EH, Suganuma T, Nilsson T (1998) Recycling of golgi-resident glycosyltransferases through the ER reveals a novel pathway and provides an explanation for nocodazole-induced Golgi scattering. *J Cell Biol* **143:** 1505–1521

Sutterlin C, Hsu P, Mallabiabarrena A, Malhotra V (2002) Fragmentation and dispersal of the pericentriolar Golgi complex is required for entry into mitosis in mammalian cells. *Cell* **109:** 359–369

Tagaya M, Furuno A, Mizushima S (1996) SNAP prevents Mg^{2+}-ATP-induced release of *N*-ethylmaleimide-sensitive factor from the Golgi apparatus in digitonin-permeabilized PC12 cells. *J Biol Chem* **271:** 466–470

Takeda M, Dohmae N, Takio K, Arai K, Watanabe S (2001) Cell cycle-dependent interaction of Mad2 with conserved Box1/2 region of human granulocyte–macrophage colony-stimulating factor receptor common βc. *J Biol Chem* **276:** 41803–41809

Tang BL, Zhang T, Low DY, Wong ET, Horstmann H, Hong W (2000) Mammalian homologues of yeast sec31p. An ubiquitously expressed form is localized to endoplasmic reticulum (ER) exit sites and is essential for ER–Golgi transport. *J Biol Chem* **275:** 13597–13604

Waters MG, Clary DO, Rothman JE (1992) A novel 115-kD peripheral membrane protein is required for intercisternal transport in the Golgi stack. *J Cell Biol* **118:** 1015–1026

Wickner W, Haas A (2000) Yeast homotypic vacuole fusion: a window on organelle trafficking mechanisms. *Annu Rev Biochem* **69:** 247–275

Williams BC, Gatti M, Goldberg ML (1996) Bipolar spindle attachments affect redistributions of ZW10, a *Drosophila* centromere/kinetochore component required for accurate chromosome segregation. *J Cell Biol* **134:** 1127–1140

Williams BC, Goldberg ML (1994) Determination of *Drosophila* zw10 protein localization and function. *J Cell Sci* **107:** 785–798

Williams BC, Karr TL, Montgomery JM, Goldberg ML (1992) The *Drosophila l*(1) *zw10* gene product, required for accurate mitotic chromosome segregation, is redistributed at anaphase onset. *J Cell Biol* **118:** 759–773

Wojcik E, Basto R, Serr M, Scaerou F, Karess R, Hays T (2001) Kinetochore dynein: its dynamics and role in the transport of the Rough deal checkpoint protein. *Nat Cell Biol* **3:** 1001–1007

Xiao J, Liu C-C, Chen P-L, Lee W-H (2001) RINT-1, a novel rad50-interacting protein, participates in radiation induced G_2/M checkpoint control. *J Biol Chem* **276:** 6105–6111

Yamaguchi T, Dulubova I, Min SW, Chen X, Rizo J, Südhof TC (2002) Sly1 binds to Golgi and ER syntaxins via a conserved N-terminal peptide motif. *Dev Cell* **2:** 295–305

Yamaguchi T, Yamamoto A, Furuno A, Hatsuzawa K, Tani K, Himeno M, Tagaya M (1997) Possible involvement of heterotrimeric G proteins in the organization of the Golgi apparatus. *J Biol Chem* **272:** 25260–25266

Zaal KJ, Smith CL, Polishchuk RS, Altan N, Cole NB, Ellenberg J, Hirschberg K, Presley JF, Roberts TH, Siggia E, Phair RD, Lippincott-Schwartz J (1999) Golgi membranes are absorbed into and reemerge from the ER during mitosis. *Cell* **99:** 589–601

